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HEMOGLOBIN-BASED BLOOD SUBSTITUTES: CHARACTERIZATION OF FIVE PYRIDOXAL 5′-PHOSPHATE DERIVATIVES OF HEMOGLOBIN*

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SUMMARY

Six fractions (I–VI) of pyridoxal 5′-phosphate (PLP) hemoglobin (Hb), prepared by the method of De Venuto and Zegna [J. Surg. Res., 34 (1983) 205] have been isolated and purified by anion-exchange high-performance liquid chromatography (HPLC). Total phosphate analyses indicate that I is HbA, II and III are double-labelled, IV and V are tetra-labelled and VI contains 6 mol of phosphorus per mol of hemoglobin. The purified components have been resolved into their α and β chains by preparative reversed-phase HPLC using a macroporous C_4 support. Phosphate analyses indicate that the β chains of II, III and IV each contain one phosphate per chain while the β chains of V and VI each contain two phosphates. The α chains of IV and VI were found to be monophosphate-labelled. Reversed phase HPLC analysis of the tryptic peptides of the β chains indicates that the label is bound exclusively to the 1-valine residue in II, III and IV while both the 1-valine and the 82-lysine are labelled in V and VI. Similarly, modification of the 1-valine residue of the α chains of IV and VI was detected. Components II and III have the same molecular formula. Evidence is presented which shows that they are interconvertable and that they correspond to the PLP_2-Hb species and component V is Benesch's PLP_4-Hb [J. Biol. Chem., 257 (1983) 1320 and references cited therein]. Component IV is III with one additional PLP per α chain and similarly VI is V with monolabelled α chains. Both IV and VI are hitherto unknown pyridoxal 5′-phosphate derivatives of hemoglobin. The oxygen affinities of the components were determined and were found to decrease with increases in the extent of pyridoxal 5′-phosphate labelling. This effect, however, reaches a maximum in V since VI which contains 6 mol of phosphate per mol of hemoglobin has a higher affinity than V.

INTRODUCTION

Pyridoxal 5′-phosphate (PLP)-labelled hemoglobin (Hb) has attracted considerable attention both as a potential hemoglobin-based blood substitute and as

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the precursor of other materials prepared with the same intended application [1–4]. Benesch et al. [5] initially prepared PLP-Hb by reducing the imines formed from PLP and Hb under anaerobic conditions with sodium borohydride. As well, two components of the product mixture were isolated and characterized by Benesch et al. [5]. In order to increase the yield of PLP-labelled products, others have modified Benesch's preparative procedures by modifying Hb in the presence of four molar equivalents of PLP [6,7]. The effects of these changes on the composition of the modified products, however, have not been determined. To fill this void, we have recently resolved these mixtures via anion-exchange high-performance liquid chromatography (HPLC) [8] and have found that the mixtures are comprised of six components. Fig. 1 represents a typical analytical chromatogram and also illustrates the numbering scheme used to describe the components. Briefly, the components were isolated and then purified using a combination of conventional and monobead anion-exchange chromatographies. Mol phosphate/mol Hb ratios were determined for each of the components and these results indicated that I was unlabelled, II and III were double-labelled, IV and V were tetra-labelled and VI contained six PLPs per Hb. We now report the isolation of the α and β globin chains of each of the components by reversed-phase HPLC (RP-HPLC). Mol phosphate/mol globin ratios have been evaluated. The label loci in each of the globins have been determined via RP-HPLC analysis of their tryptic peptides. The oxygen dissociation curves of the components have been determined and these results are discussed in the context of the assigned structures.

EXPERIMENTAL

Unless otherwise stated, all buffers and reagents were obtained in the highest available purity from Sigma (St. Louis, MO, U.S.A.) or BDH (Toronto, Canada). Outdated whole blood was provided by the Canadian Red Cross. Hemoglobin A (HbA) was isolated by the method of Williams and Tsay [9] from outdated red blood cells. HbA was further purified by affinity chromatography [10]. Ethyleneimine was prepared from 2-aminoethyl hydrogen sulphate by the method of Reeves et al. [11]. All RP-HPLC buffers were filtered through 0.22-μm membranes, sonicated, sparged with helium and then stored under helium during the chromatographic operations. RP-HPLC samples were sonicated immediately prior to injection.

Isolation of the component of PLP-Hb

PLP-Hb was prepared from stroma-free hemoglobin and four equivalents of pyridoxal 5′-phosphate by the method of De Venuto and Zegna [6]. The carbon monoxide derivatives of six fractions of the mixture were isolated and purified using a combination of preparative conventional (Pharmacia Q Sepharose Fast Flow) and monobead (Pharmacia Mono Q HR 16/10) anion-exchange chromatography as described [8]. The components are numbered in order of decreasing anionic mobility (see Fig. 1) in the analytical chromatogram of the reaction mixture.

TABLE I

PHOSPHATE ANALYSES AND OXYGEN BINDING PROPERTIES OF THE COMPONENTS OF PYRIDOXAL 5′-PHOSPHATE HEMOGLOBIN

Component	Mol phosphate/ mol Hb	Mol phosphate/ mol β globin	Mol phosphate/ mol α globin	P_{50} (mmHg)
I	0.1 (±0.2)	0.2 (±0.3)	0.0 (±0.3)	6
II	2.1	1.0	0.0	17
III	1.9	1.0	−0.2	17
IV	4.1	1.1	0.8	25
V	3.9	1.8	−0.1	37
VI	6.1	2.0	1.2	31
(HbA)	0	0	0	7

RP-HPLC globin separations

The HPLC separations were performed with a Waters (Waters Assoc., Bedford, MA, U.S.A.) HPLC system consisting of a U6K universal injector, a 680 automated gradient controller, two 610 pumps, a 480 Lamda Max LC detector and a Hewlett-Packard (Hewlett-Packard Canada, Mississauga, Canada) 3390-A integrator. For analytical scale separations, a Vydac (Separations Group, Hesperia, CA, U.S.A.) 214TP54 column (5 μm, 250×4.6 mm) was used under conditions similar to those reported by Shelton et al. [12]. Buffer A was 20% aqueous acetonitrile plus 0.1% trifluoroacetic acid (TFA) and buffer B was 60% aqueous acetonitrile containing 0.1% TFA. The samples, typically 10–15 μg in 100 μl of the initial buffer mixture (48% B) were eluted at a flow-rate of 1.0 ml/min with a linear gradient of 48% B to 60% B over 60 min.

Preparative separations were carried out on a Vydac 214TP510 column (5 μm, 250×10 mm) using the same buffers as above. At a flow-rate of 2.5 ml/min, 1.0 mg of sample in 1.0 ml of the initial mixture was eluted for 10 min with 48% buffer B followed by a linear gradient of 48% B to 60% B over 30 min and then 60% B over 10 min. The effluent was monitored at 220 nm and all fractions with absorbances greater than 0.2 were collected with a Pharmacia (Pharmacia, Uppsala, Sweden) Frac-100 fraction collector. The peaks corresponding to the globins were pooled, evaporated to a third of their initial volume and then lyophilized. The columns were maintained, cleaned and stored as recommended by Shelton et al. [12].

Phosphate analysis

After determining the concentration of solutions of the purified components from their absorbance of 540 nm ($\epsilon_{heme} = 14.3\ cm^{-1}\ mM^{-1}$) [13], aliquots were analyzed for total phosphate by the method of Ames and Dubin [14] as described [8]. The results, expressed as mol phosphate/mol hemoglobin are listed in Table I and represent the average of three sets of triplicate analyses. The ratios of mol phosphate/mol globin were determined from approximately 0.1% solutions of the globins in 0.4% TFA. Aliquots were analyzed for total protein using a method similar to that described by Bradford [15]. Aliquots (25 μl) were diluted to 1.0

ml with 0.4% TFA and then treated with 1.0 ml of Bradford's reagent (Pierce, Rockford, IL, U.S.A.). The absorbance at 595 nm was obtained and yielded the total globin value by comparison with a working curve determined from authentic globin samples. Aliquots of the globin solutions (200 μl) were lyophilized and then assayed for total phosphate as above. The results reported in Table I represent the averages of three analyses.

Aminoethylation of the globins

The globins were aminoethylated using a scaled down version of the procedure described by Acharya and Manning [16]. The globin (ca. 500 μg) was dissolved in 200 μl of a buffer comprised of 8.0 *M* urea, 3.0 *M* Tris and 0.1 *M* EDTA at pH 8.6, treated with 2-mercaptoethanol (5 μl) and then left at room temperature for 45 min. Ethyleneimine (7.5 μl) was added. After 10 min, a second aliquot of ethyleneimine (7.5 μl) was added and then the mixture was allowed to stand at room temperature for 30 min. The mixture was desalted by elution through a column (10×1.0 cm) of Sephadex G-25 Superfine with 0.2 *M* acetic acid at a flow-rate of 0.2 ml/min. The effluent was monitored at 280 nm and the fractions containing the first peak (aminoethylated globin, retention time 12 min) were pooled and then lyophilized.

Tryptic peptides

The aminoethylated globin (500 μg) was suspensed in 20 m*M* ammonium carbonate, pH 8.0 (250 μl) which contained TPCK-trypsin (10 μg). After 4 h of incubation at 37°C, the mixture was treated with 2.0 *M* acetic acid (50 μl) and then lyophilized.

RP-HPLC resolution of the tryptic peptides

The peptides were resolved by the method of Acharya et al. [17] on a Whatman Partisil 10-ODS-2 column (5 μm, 250×4.6 mm) installed in the above-described Waters HPLC system. Buffer A was 0.1% aqueous TFA and buffer B was 0.1% TFA in acetonitrile. At a flow-rate of 1.0 ml/min, the peptide sample (450 μg) in 95% A (100 μl) was eluted with a linear gradient of 5% B to 40% B (for the β peptides) or 50% B (for the α peptides) over 80 min. The effluent was monitored at 210 nm (1.0 a.u.f.s.) and fractions were collected at 2.0-min intervals. The fluorescence of the fractions at 410 nm, when irradiated at 325 nm, was measured with a Perkin-Elmer 650 10 M fluorescence spectrophotometer.

Oxygen dissociation curves

The oxygen dissociation curves were obtained from 3.5% solutions of the purified components in 50 m*M* Bis–Tris with an Aminco Hemo-O-Scan oxygen dissociation analyzer. The samples were stripped of carbon monoxide during their equilibration in the Hemo-O-Scan [18]. The P_{50} values were read directly from the curves.

Anion-exchange HPLC analysis of the interconversion of components II and III

Solutions of II and III (3.0%) in 20 m*M* Tris–HCl pH 8.0 were diluted to 1.0, 0.2 and 0.3% with the same buffer. The solutions were saturated with carbon

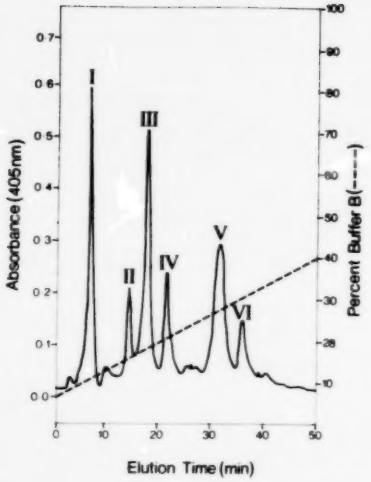


Fig. 1. Analytical anion-exchange HPLC profile of 500 μg of the carbon monoxide-saturated pyridoxal 5′-phosphate–hemoglobin reaction mixture eluted at ambient temperature from a Mono Q HR 5/5 column (50 × 5 mm) at a flow-rate of 1.0 ml/min. Buffer A is 20 m*M* Tris–HCl and buffer B is 0.5 *M* sodium chloride in buffer A.

monoxide, left at room temperature for 24 h and then analyzed by anion-exchange HPLC using a Pharmacia Mono Q HR 5/5 column as previously described [8].

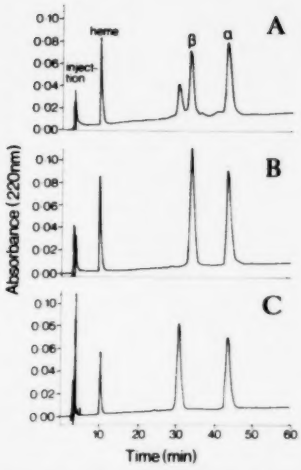
RESULTS

PLP-Hb preparation and component isolation

PLP-Hb was prepared by anaerobic sodium borohydride reduction of the adducts formed from stroma-free hemoglobin and pyridoxal 5′-phosphate by the method of De Venuto and Zegna [6]. As previously described [8], the reaction mixture was saturated with carbon monoxide and then resolved via HPLC (Pharmacia Mono Q) into six fractions as shown in Fig. 1. With a combination of preparative conventional (Pharmacia Q Sepharose Fast Flow) and monobead anion-exchange chromatography (Pharmacia Mono Q HR 16/10), specimens of each component were isolated and purified. All gave single peaks on HPLC analysis. Mol phosphate/mol hemoglobin ratios were determined for each of the components and the results are listed in Table I (see first column).

RP-HPLC globin separations

The recently reported high-resolution PR-HPLC separations of hemoglobin and its variants into α and β chains [12] led us to attempt analogous separations with the purified components of PLP-Hb. Using methodology similar to that developed by Shelton et al. [12], the PLP-Hb reaction mixture was resolved on an analytical scale by RP-HPLC with a macroporous C_4 Vydac TP54 column using a linear gradient of TFA containing aqueous acetonitrile eluents. As shown in Fig. 2A, the mixture is cleanly resolved into peaks ascribed to hemes, two types



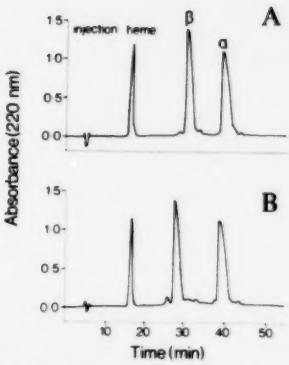


Fig. 2. Analytical reversed-phase HPLC profiles of the PLP-Hb reaction mixture (A), purified component II (B) and purified component V (C). The samples (15 μg in 100 μl of the initial buffer mixture) were eluted from a Vydac 214TP54 column (250×4.6 mm) at a flow-rate of 1.0 ml/min. Buffer A is 0.1% TFA in 20% acetonitrile and buffer B is 0.1% TFA in 60% acetonitrile. A linear gradient of 48% B to 60% B over 60 min was used.

Fig. 3. Preparative reversed-phase chromatograms of components III (A) and V (B) of the PLP-Hb mixture obtained from 1.0 mg of the samples in 1.0 ml of the initial buffer mixture with a Vydac 214TP510 column (250×10 mm) at a flow-rate of 2.5 ml/min. Buffer A is 0.1% TFA in 20% acetonitrile and buffer B is 0.1% TFA in 60% acetonitrile. The elution was 48% B for 10 min, a linear gradient of 48% to 60% B over 30 min and then 60% B for 10 min.

of β globins and an α globin. These assignments have been made by comparing chromatogram Fig. 2A with those reported by Shelton et al. [12]. The HPLC PLP-Hb reaction mixture (see Fig. 2A) clearly indicates the presence of two types of β chains and, therefore, demonstrates that at least one form of PLP labelling of the β chains results in a change in retention time. The components from Fig. 1 were each analyzed and representative chromatograms are shown in Fig. 2B and Fig. 2C. Components I, II, III and IV gave chromatograms indistinguishable from that obtained from HbA. Those of V and VI both contain the more mobile β globin and are themselves indistinguishable. None of the components gave chromatograms exhibiting both types of β peaks.

Accordingly, preparative-scale HPLC resolution of the globins was examined using a Vydac 214TP510 column. Fig. 3 shows the chromatograms obtained from 1.0-mg quantities of fractions III (Fig. 3A) and V (Fig. 3B). Noteworthy is the relatively small loss in resolution that has accompanied the scale-up of the separations (compare Fig. 2 with Fig. 3). Indeed, baseline globin separations could be obtained under the preparative conditions with column load of up to 4 mg. As with the analytical chromatograms, the preparative chromatograms of HbA, I, II, III an IV are indistinguishable as are those of V and VI. The latter pair again both contain the more mobile β globins. The globins of each component, after

isolation, were analyzed using the HPLC method and shown to consist of a mono peak.

Phosphate/globin ratios

In order to determine which of the globins in the components were phosphate-labelled and the stoichiometry of that labelling, mol of phosphate to mol of globin ratios were obtained for each of the purified globins. The resulting mol phosphate/mol globin ratios listed in Table I clearly show that component I is unlabelled as are the α globins of II, III and V. The β globins of II, III and IV each contain an average of one PLP as do the α globins of IV and VI. The β globins of V and VI each bear an average of two labels. This suggests that the isolated components are single species that can be described by a single molecular formula. Comparison of these results with those of the HPLC analyses indicates that monolabelling the α and β chains produces no detectable change in their mobility. Double-labelled β chains, however, show increased mobility.

Tryptic peptide analysis

In order to determine the precise location of the labels on the chains, the globins were aminoethylated and then digested with TPCK-trypsin. The resulting peptides were resolved by HPLC using the method of Acharya et al. [17] and of Benesch et al. [5]. Also following Benesch's example, the presence of PLP groups on modified peptides was confirmed by the detection of fluorescence in the modified peptides. The chromatogram of the tryptic peptides from the β chains of component I is shown in Fig. 4A and is indistinguishable from one obtained from HbA. With the exception of a reversal in the elution order of the T_9 and T_5 (retention time 64 min) peptides, the chromatogram of Fig. 4A contains the same peaks in the same elution order and relative intensities as does the chromatogram of the tryptic peptides from HbA reported by Acharya et al. [17]. These minor differences are attributable to variations in the gradient preparation and, therefore, the peptide assignments of Acharya et al. [17] have been assumed. The tryptic peptide chromatograms of β globins obtained from II, III and IV are indistinguishable as are those from V and VI. Representative chromatograms of these two types of β chain peptides are shown in Fig. 4B and C. Relative to the T_1 peptide of I (Fig. 4A, the T_1 peptide of II (Fig. 4B) shows a significant increase in the retention time. Furthermore, the modified T_1 peptide exhibits the anticipated fluroescent activity (see shaded peak of Fig. 4B). These are precisely the changes observed by Benesch et al. [5] in their analysis of the PLP-1-valine labelled tryptic peptides. It may, therefore, be concluded that the β chains of components II, III and IV are all labelled exclusively on the N-terminal 1-valine residue. The chromatograms of the β chain peptides of V and VI contain the same modified T_1 peptide and also exhibit changes in the T_9 and T_{10} peptides (Fig. 4C). The latter pair appear as one broad peak of decreased mobility which shows fluorescent activity (see shaded peaks in Fig. 4C). Again, this is exactly what has been observed by Benesch et al. [5] in their analysis of the PLP-1-valine, PLP-82-lysine double-labelled β chains. Also noteworthy is that the relative fluores-

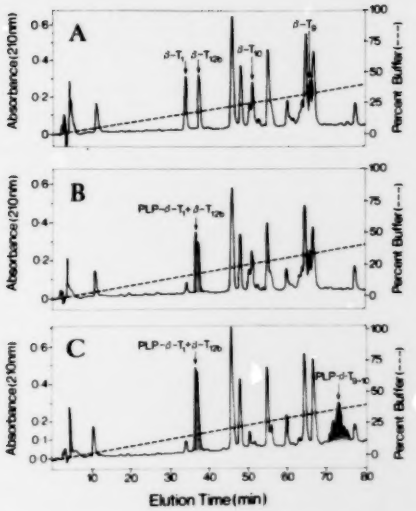


Fig. 4. Reversed-phase chromatograms of the tryptic peptides of the β globins of the PLP-Hb components I (A), II (B) and VI (C) obtained from 400 μg of the samples with a Whatman Partisil 10 ODS-2 column (250 × 4.6 mm) at a flow-rate of 1.0 ml/min. Buffer A is 0.1% aqueous TFA and buffer B is 0.1% TFA in acetonitrile. The shaded areas depict the fluorescent peptides (410 nm emission with 325 nm irradiation).

cences of the two modified peptides are comparable to those observed by Benesch et al. [5] (Table II). We therefore concluded that components V and VI both contain β globins with PLPs covalently bound to the 1-valine and 82-lysine residues.

The tryptic peptide chromatograms from the unmodified α chains of HbA, I, II, III and V were indistinguishable (see Fig. 5A). These chromatograms are sufficiently close reproductions of the chromatogram of the tryptic peptides from

TABLE II

NORMALIZED RELATIVE FLUORESCENT INTENSITIES OF THE LABELLED TRYPTIC PEPTIDES OF THE COMPONENTS OF PYRIDOXAL 5′-PHOSPHATE HEMOGLOBIN

Peptide components	PLP-β-T_1*	PLP-β-T_9+T_{10}*	PLP-α-T_1**
II	106	–	–
III	103	–	–
IV	90	–	100
V	100	49	–
VI	94	44	97

*The fluorescent intensities of the β chains were normalized to PLP-β-T_1 peptide of VI by comparing the peak areas of the T_3, T_{13}, T_{14} and T_{11} peptides (retention times 46–50 min) of the RP-HPLC profiles of each component (see Fig. 5).

**The relative fluorescent values were normalized using the peak area T_6 peptide (retention time 46 min) shown in Fig. 6.

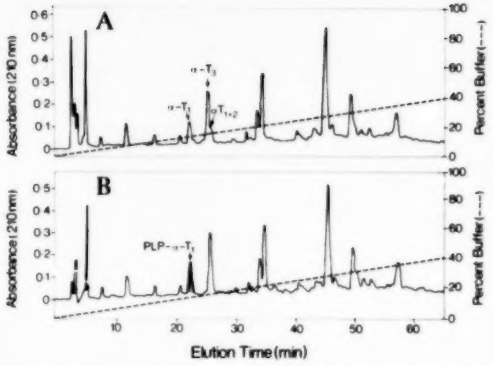


Fig. 5. Reversed-phase chromatograms of the tryptic peptides of the α globins of III (A) and IV (B) obtained as described in Fig. 4. The shaded area depicts the fluorescent peptide.

the α chain of HbA reported by Acharya et al. [17] that amino acid analysis was unnecessary for the peptide assignments. Components IV and VI gave indistinguishable chromatograms (Fig. 5B) which in addition were very similar to that from I (see Fig. 5A). The only detectable difference is a slight decrease in the retention time of the T_1 peptide (0.5 min) and the disappearance of the T_1+T_2 peak in the chromatograms from the labelled globins. Fluroescent activity, however, is only observed from the T_1 peptides of IV and VI (see shaded peak in Fig. 5B) and, therefore, confirms the modification of T_1 peptide with PLP. Since the only available PLP binding site on this peptide is the 1-valine amino group, it can be concluded that the α chains of IV and VI are labelled exclusively on their N-terminal 1-valines.

Concentration dependence of the anion-exchange HPLC of components II and III

The most striking feature of the results of the tryptic peptide analyses is that II and III are comprised of the same types of α and β chains. Subsequent HPLC analysis of a specimen of II which had been stored at 4°C for several months indicated the presence of an appreciable amount of III. Similarly, a stored specimen of III was found to contain a significant amount of II. This phenomenon suggests that II and III are interconvertable. Thus a simple experiment was devised to test this hypothesis. A specimen of II was diluted to several known concentrations and the resulting solutions, after 24 h of incubation at ambient temperature, were analyzed by HPLC. The resulting chromatograms are shown in Fig. 6. A set of solutions of III with concentrations identical to the above set, when manipulated in the same manner, gave a set of chromatograms indistinguishable from those shown in Fig. 6. These results show that at low concentrations, III converts to II (Fig. 6A) and at high concentrations, II becomes III (Fig. 6C). A mixture of both is formed from either II or III at intermediate concentrations (Fig. 6B). This result clearly indicates that II and III are linked by an equilibrium and that

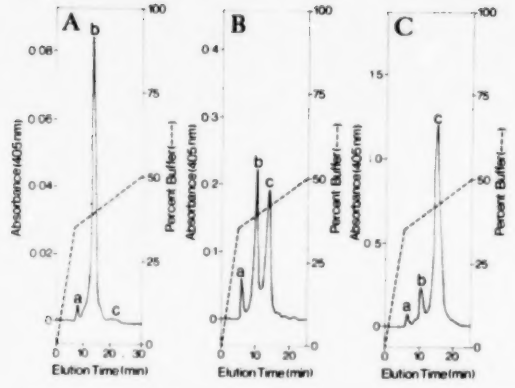


Fig. 6. Analytical anion-exchange chromatograms of carbon monoxide-saturated PLP-Hb component II obtained with a Mono Q HR 5/5 column (50×5 mm) at a flow-rate of 1.0 ml/min. Buffer A is 20 m*M* Tris-HCl and buffer B is 0.25 *M* sodium chloride in buffer A. A was obtained from a specimen of II which had been stored at 23°C for 24 h as a 0.03% solution in buffer A. B and C are those obtained after storage as 0.2 and 1.0% solutions, respectively. The labels a, b and c denote, respectively, the elution positions of HbA (peak I) and of freshly isolated specimens of peaks II and III shown in Fig. 1.

the equilibrium is concentration-dependent. Furthermore, that II and III are isolable indicates that, relative to the HPLC separation, the equilibration process is slow.

Oxygen dissociation curves

The oxygen dissociation curves of the components were obtained and are shown in Fig. 7. As anticipated, the curves from HbA and III are indistinguishable from those of I and II, respectively, and, therefore, are not shown. The P_{50} values were read directly from the curves and are listed in Table I (see fourth column). Examination of these data indicated that a general decrease in oxygen affinity is observed with increasing labelling stoichiometry. The effect, however, reaches a maximum in component V, as VI shows a slight reduction in P_{50}.

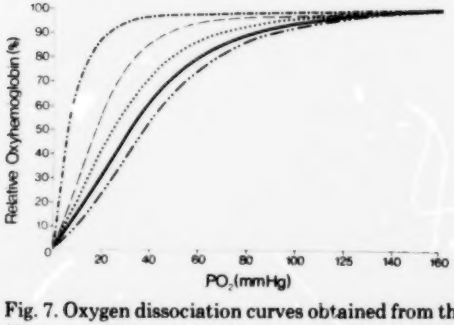


Fig. 7. Oxygen dissociation curves obtained from the PLP-Hb reaction mixture component I (- · -), III (- - -), IV (.....), V (- ·· -) and VI (——) with an Aminco Hemo-O-Scan oxygen dissociation analyzer. The samples were analyzed as 3.5% solutions in 50 m*M* Bis-Tris pH 7.0 at 37°C.

DISCUSSION

In the absence of the intraerythrocytic allosteric effector, diphosphoglycerate (DPG), stroma-free hemoglobin has a high oxygen affinity and is, therefore, ineffective as an oxygen transporting blood substitute [19,20]. By covalently labelling the DPG binding site with PLP, Benesch et al. [20], successfully reduced the oxygen affinity of Hb to that of red blood cells. Consequently, PLP-Hb has been extensively studied as a blood substitute [6,7] and, more recently, has been used as the starting material for polymerized PLP-Hb (poly-PLP-Hb) which is also a potential blood substitute [1–4]. In the present study, we show that PLP-Hb mixtures typical of those used in the preparation of poly-PLP-Hb when analyzed by HPLC (Fig. 1), are more complex than those reported by Benesch et al. [5]. In contrast, Benesch et al. [5] resolved their PLP-Hb on a phosphocellulose column and isolated two main reaction products: PLP_2-Hb and PLP_4-Hb. These were characterized and found to have PLPs bound to the 1-valine residues of the β chains in the former and to both the β-1-valines and β-82-lysines in the latter. Several laboratories have modified Benesch's preparative procedures in order to facilitate large-scale production of PLP-Hb [6,7]. The effects of these changes on the composition of the product mixtures, however, is unknown. Also, subsequent studies of PLP-Hb or its gluteraldehyde cross-linked polymer, poly-PLP-Hb, have been vulnerable to batchwise variations in the PLP-Hb preparations. Clearly, a precise description of the composition of these mixtures as well as a convenient analytical method for compositional determinations would be highly desirable.

In the selection of ion-exchange monobead HPLC columns for this study, we used both Mono Q and Mono S (Pharmacia) and found them to have similar resolving power. However, for the resolution of the PLP-Hb components, we found Mono Q was more desirable because of the basic elution conditions used, which minimized the formation of methemoglobin and made possible subsequent functional studies. We have also used Q-Sepharose Fast Flow to pre-purify the PLP–Hb reaction mixture [8]. The components identified in Fig. 1 were then purified from selected fractions by a preparative Mono Q column. To ensure purity, a second preparative Mono Q was also run. These components were analyzed for their phosphate/heme ratios. The ratios indicated that I is unlabelled, II and III contain two PLPs per Hb, IV and V are tetra-labelled and VI contains six phosphates per mol of Hb. Our results demonstrate that the mixtures contain three new PLP derivatives of Hb in addition to PLP_2-Hb and PLP_4-Hb reported by Benesch et al. [5].

In the present study, the six isolated components of PLP-Hb have been characterized using the following sequence which is similar to that developed by Benesch et al. [5]. The components were each split into their α and β chains via preparative HPLC. This method was used in lieu of the classical procedures [21] because it offered advantages in smaller scale, product purity and convenience [12]. Determination of phosphate/globin ratios established the PLP labelling stiochiometry on a globin basis for each of the components (see Table I). To determine the position of PLP labelling on each of the globins, the globins were

TABLE III

DIMER COMPOSITION OF THE COMPONENTS OF PYRIDOXAL 5'-PHOSPHATE HEMOGLOBIN

Components	$\alpha\beta$ Dimer structure	
I, HbA	α (unmodified)	β (unmodified)
II, III	α (unmodified)	β LP-1-Val
IV	α PLP-1-Val	β PLP-1-Val
V	α (unmodified)	βPLP-1-Val
		β PLP-82-Lys
VI	αPLP-1-Val	β PLP-1-Val
		β PLP-82-Lys

aminoethylated and then enzymatically hydrolyzed with PTCK-trypsin thereby cleaving all C-terminal lysine, arginine and aminoethylated cystine linkages. The resulting peptides were separated by HPLC (Figs. 4 and 5) and the labelled peptides were identified by the detection of changes in their HPLC mobility and of fluorescent activity attributable to the PLP moiety. The results of these experiments are summarized in Table III. The oxygen dissociation curves (Fig. 7) were obtained from the components and the oxygen affinities (P_{50} values, see Table II) generally showed an inverse correlation with the extent of modification. The only exception in the P_{50} of VI which is less than that of V.

Comparison of the structural results shown in Table III with those of Benesch et al. [5] indicated that I is unmodified HbA, II and III both correspond to Benesch's PLP_2-Hb and V is PLP_4-Hb. Components IV and VI are mono-PLP-labelled α-globin derivatives of III and V. IV and VI have not been previously characterized.

An additional similarity between the structures reported in Table III and those deduced by Benesch et al. [5] is that in all cases, the tetramers formed from the globins consist of pairs of identical $\alpha\beta$ subunits. Benesch et al. [5] have referred to such species as being symmetrical tetramers, as compared to tetramers comprised of non-identical $\alpha\beta$ subunits as being asymmetrical. For example, a PLP_2-Hb made from the subunits of III is symmetrical, whereas a PLP_2-Hb made from an $\alpha\beta$ subunit from I and a subunit from V is asymmetrical. Furthermore, symmetrical tetramers can, via reversible subunit dissociation, form asymmetric tetramers. For example, the asymmetric PLP_2-Hb mentioned above can disproportionate into I and V. Similarly, a mixture of I and V, both symmetrical tetramers, will, at equilibrium, contain the asymmetric PLP_2-Hb mentioned above. Benesch et al. [5] have shown that ion-exchange chromatographic separations are slow relative to subunit dissociation and, consequently, the dissociation equilibria are driven during elution to maximize charge separation. Chromatographically isolated components, therefore, will consist only of symmetric species even if asymmetric tetramers are present in the original mixture. However, under anaerobic conditions, subunit dissociation is suppressed. Accordingly, the presence of asymmetric tetramers in the reaction mixture was detected by anaerobic

electrophoresis [5]. It follows then that the isolation of exclusively symmetrical tetramers in the present study does not indicate that they are the only species present in the mixture. Rather, it suggests that relative to subunit dissociation, the HPLC separations are also slow and that they serve to drive the dissociation equilibria to maximum charge separation.

A surprising feature of the structural assignments is that II and III are both comprised of 1-valine-labelled β-globins and unlabelled α-globins (Table III). Subsequent HPLC analysis indicated that the two were interconvertible (Fig. 6). At high concentrations, II slowly became III and at low concentrations III converted to II. At intermediate concentrations, identical mixtures of II and III were obtained from both. This indicated that II and III were in equilibrium. The most obvious reaction scheme that satisfies this requirement is a simple dissociation of tetramer into $\alpha\beta$ subunit wherein III is the tetramer and II is the $\alpha\beta$ dimer. Since the formation of III from II is a second-order process while the formation of II from III is a first-order process, this explanation accounts for the concentration dependence of the equilibrium and fulfills the compositional requirements as well. The resolution of the components in the present study, however, requires that the equilibrium be slow relative to the HPLC separation. This contradicts the previous conclusions derived from the exclusive observation of symmetrical products [5]. In the absence of further information, no conclusion regarding its mechanism can be made. Furthermore, the potential of the ion-exchange chromatographic method to modify the equilibrium ratio of II to III makes it less than ideal for detailed study of this phenomenon. The results presented herein simply show that II and III are comprised of identical $\alpha\beta$ dimers and that the components are linked by an equilibrium process.

The α-PLP-labelled components, IV and VI, have not been observed by Benesch et al. [5]. Evidently, IV and VI are the result of increased reactivity of the labelling reaction that occurs as a consequence of changes in the reaction conditions. They may also exist in Benesch's mixtures as minor components. Of interest to blood substitute researchers are the oxygen binding properties of the labelled components (IV and VI). The oxygen dissociation curve of IV is less right-shifted than PLP_4-Hb (V) and more right-shifted than PLP_2-Hb (III). The PLP labelling of the α chain in IV, therefore, imparts the desired effect in further reducing the oxygen affinity of Hb. Conversely, when α chain of V is PLP-labelled to give VI, an increase in oxygen affinity is observed (see Table I). The two additional α PLP labels in VI have an adverse effect on the allosteric reduction of the oxygen affinity of hemoglobin. Presumably, steric crowding in the DPG binding site in VI relative to V is responsible for the observed reduction. A noteworthy feature of the oxygen affinities of the α-PLP-labelled components, i.e. IV and VI, is that they both lie between the affinities of the double- and tetra-PLP-labelled components III and V. Thus their presence in the PLP–Hb reaction mixtures will not increase the average oxygen affinity of PLP-Hb-based blood substitutes.

The HPLC analytical method, as we have previously shown, is simple, fast, precise and highly reproducible. The results of the present study, however, indicate that, as a consequence of ion-exchange-driven disproportionation, the observed product composition will not directly correspond to the composition of

the analyzed mixture. All asymmetric species will appear as symmetrical tetramers. The method does, however, provide a means with which to obtain an inventory of the relative amounts of each type of $\alpha\beta$ dimer in the PLP–Hb mixtures (see Table III). These values should be useful in the quality control for the scale-up production of PLP-Hb as the starting material for PLP-Hb-based blood substitute.

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